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Identification and epidemiological characterization of *Streptococcus uberis* isolated from bovine mastitis using conventional and molecular methods

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In the present study 130 *S. uberis* strains and one *S. parauberis* strain isolated from bovine milk samples of 58 different farms of various locations in Hesse, Germany, as well as two reference strains of each species were comparatively investigated for cultural, biochemical, serological and molecular properties. All *S. uberis* strains produced the enzyme β-D-glucuronidase, while the *S. parauberis* strains were negative. The *S. uberis* and *S. parauberis* 16S rRNA genes were amplified by polymerase chain reaction and subsequently digested with the restriction enzymes *Rsa*I and *Ava*II yielding species-specific restriction patterns. Both species were additionally identified by amplifying species-specific parts of the genes encoding the 16S rRNA, the 23S rRNA and the 16S-23S rDNA intergenic spacer region, respectively. The CAMP factor gene *cfu*, a potential virulence factor of *S. uberis*, was amplified, corresponding to a phenotypically positive CAMP-reaction, using *cfu*-specific oligonucleotide primers. In addition the streptokinase/plasminogen activator encoding genes *skc*/*pau*A, a second potential virulence factor, could be amplified for 126 of the 130 *S. uberis* but not for *S. parauberis*. A DNA fingerprinting of *S. uberis* strains, performed by macrorestriction analysis of their chromosomal DNA by pulsed-field gel electrophoresis, revealed that most of the isolates were not related to each other. However, identical DNA patterns were noted for some of the isolates within different quarters of an individual cow and also for different cows within the same farm. The generally unrelated DNA patterns indicated that *S. uberis* is a pathogen with multiple environmental habitats and that infections are caused by a great variety of strains.

Key words: *Streptococcus uberis*, *Streptococcus parauberis*, 16S rDNA, 23S rDNA, 16S-23S rDNA intergenic spacer region, CAMP factor gene *cfu*, *skc*/*pauA* genes

Introduction

Streptococcus uberis is world wide known as an environmental pathogen responsible for a high proportion of cases of clinical, mostly subclinical mastitis in lactating cows and is also the predominant organism isolated from mammary glands during the nonlactating period [37].

S. uberis differs from other mastitis-causing streptococci in that it can also be isolated from the udder surface, from other sites on the body of cows and also from the cows environment. The most important reservoirs for infections of the mammary gland parenchyma appears to be the skin and the udder surface [35,52]. *S. uberis* can also be isolated from numerous sites including belly, lips, teats, urogenital tract, tonsils, rectum, rumen, nostrils, eye, poll, chest, sacrum, caudal folds and feces [15,18,42,44,57,63]. In addition, *S. uberis* had been isolated in large numbers from the straw bedding of housed cattle usually during the winter housing period and from the pasture grazed by infected cattle [10].

According to Sherman [58] and Slot [61] *S. uberis* showed some similarities to bacteria of genus *Enterococcus*. However, the studies summarized by Schleifer and Kilpper-Bälz [53] and Lämmler and Hahn

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[37] revealed that *S. uberis* seems to be more related to the pyogenic group of genus *Streptococcus*. On the basis of chromosomal DNA hybridizations Garvie and Bramley [23] and Collins *et al*. [14] suggested the existence of two distinct *S. uberis* genotypes, designated as *S. uberis* type I and II. According to a proposal of Williams and Collins [68] type II *S. uberis* were classified as *S. parauberis*.

In the present study *S. uberis* and *S. parauberis* strains isolated during routine diagnostics from bovine milk samples of one region in Germany were investigated together with reference strains of both species for cultural, biochemical, serological and molecular properties. The latter included the detection of various genes by polymerase chain reaction and the determination of epidemiological relationships by pulsed-field gel electrophoresis (PFGE).

Materials and Methods

Collection and cultivation

For the present study 342 bovine milk samples from 342 quarters of 269 cows from 93 different farms were initially collected within three months from January to March 1999 at different locations in Hesse, Germany. Approximately 0.1 ml milk obtained from clinical as well as subclinical milk samples were initially plated on sheep blood agar (Oxoid, Wesel, Germany), while subclinical samples were subjected to total somatic cell count (SCC) in order to confirm the subclinical status of the collected samples. The determination of cell count was performed with the Fossomatic system (360 N. Foss Electronic A/S, Hamburg, Germany).

All bacteria suspected to belong to genus *Streptococcus* were subsequently cultivated on Columbia esculin blood agar (Merck, Darmstadt, Germany) to determine their culturing ability. The esculin-hydrolyzing cultures were further cultivated on five different selective growth media specific for enterococci. This included Citrate azide tween carbonate agar (CATC, Merck), Kanamycin esculin azide agar (KAA, Merck), Esculin bile agar (Oxoid), Chromocult enterococci agar (Merck), and Slanetz-Bartley media (Oxoid). All media were prepared, used and the results interpreted according to the manufacturers instructions. An *Enterococcus faecalis* strain, obtained from the institutes strain collection (Institute of Milk Science, Giessen University, Giessen, Germany), was used as positive control.

On the basis of the above mentioned cultural ability and growth patterns 131 isolates from 112 cows of 58 different farms affected with subclinical and clinical mastitis were further processed. The isolates were investigated together with the *S. uberis* reference strains NCDO 2038 and NCDO 2086, the *S. parauberis* reference strain NCDO 2020 and the *S. parauberis* strain 94/16. The latter, originally isolated from a diseased turbot, was kindly obtained from J. F. Fernández-Garayzábal (Faculty of Veterinary Medicine, Complutense University, Madrid, Spain) [19].

Biochemical characterization

Carbohydrate fermentation tests were determined by using phenol-red broth (Merck) containing 1% arabinose, fructose, glucose, inulin, lactose, maltose, mannitol, raffinose, ribose, saccharose, salicin, sorbitol and trehalose, respectively. Esculin hydrolysis was carried out, using Brain Heart Infusion (BHI, Merck) containing 0.1% esculin and 0.05% iron (III) citrate. For determination of sodium-hippurate hydrolysis the method described by Hwang and Ederer [29] was used. For arginine hydrolysis commercial diagnostic test tablets (Rosco, Hiss Diagnostics, Freiburg, Germany) were used as substrate. The tests were carried out as described by the manufacturer. Commercial diagnostic test tablets (Rosco, Hiss Diagnostics) were also used as substrates for determination of â-D-glucuronidase, and pyrrolidonyl aminopeptidase enzyme activities. In addition, hyaluronidase enzyme activities were investigated by cultivation of the bacteria in close proximity of a mucoid growing *S. equi* subsp. *zooepidemicus* strain, obtained from the institutes strain collection, as described by Winkle [70].

Serogrouping

Serological grouping of the cultures was performed with autoclaved extracts [47] and specific antisera of Lancefield groups A, B, C, E, G, P, U and V. The antisera were obtained from the institutes collection [36].

Other phenotypic characteristics

Synergistic CAMP-like hemolytic activities were determined together with a β-toxin producing *S. aureus* on sheep blood agar plates [37], lectin agglutination reactions with the lectin from Helix pomatia (Sigma, Deisenhofen, Germany), on microscopic slides [43]. Self-agglutinating bacterial cultures were pretreated with 5 μl trypsin (1 mg trypsin/ml PBS) for 1 hr at 37°C, washed, resuspended in PBS and subsequently used for lectin agglutination as described [43].

Genotypic characterization

The extraction of the DNA of the isolates was performed as described [28]. The gene encoding the 16S rRNA was amplified using the oligonucleotide primer ARI with the sequence 5' GAGAGTTTGATCCTGGCTCAGGA 3' [8] and the primer AmII with the sequence 5' CGGGTGTTAC AAACTCTCGTGGT 3' [3]. The oligonucleotide primers were synthesized by MWG-Biotech (Ebersberg, Germany). Restriction fragment length polymorphism

analysis (RFLP) of the amplified 16S rRNA gene was performed as recommended by Jayarao *et al*. [30] and Lämmler *et al.* [38]. The amplicon was digested for 1 hr at 37°C in a water bath in 30 µl volumes with 1µl (10 U/µl) *Rsa*I and *Ava*II (New England Biolabs, Frankfurt, Germany), respectively.

A molecular identification was additionally performed by using species-specific oligonucleotide primers for the genes encoding the 16S rRNA and 23S rRNA as well as the 16S-23S rDNA intergenic spacer region with oligonucleotide primers described previously [28]. In addition, phenotypically CAMP positive and selected CAMP-negative *S. uberis* and *S. parauberis* were investigated for CAMP factor gene *cfu*. For amplification of *S. uberis* CAMP factor gene *cfu* the oligonucleotide primers were designed according to the *cfu* sequence of *S. uberis* described by Jiang *et al*. [33]; (accession no. U34322) by using computer program OLIGO 4.0. The primer 1 had the sequence cfu-I 5' CTTTATTTTCCCCAA 3' and primer 2 the sequence cfu-II 5' ATTTCTTGGTCAA CTTGT 3'. The PCR temperature program of 30 cycles was: 92°C for 60 sec, 45°C for 1.5 min, 72°C for 1.5 min. The final cycle was followed by an extension at 72°C for 5 min.

The amplification of another potential virulence factor gene of *S. uberis* known as streptokinase/plasminogen activator gene *skc*/*pauA* was performed as described by Rosey et al. [51]; (accession no. AJ012549) and Johnsen *et al.* [34]; (accession no. AJ131604), respectively. Amplification of the gene *skc* was conducted using the oligonucleotide primer SKC-I as primer 1 with the sequence 5' CTCCTCTCCAACAAAGAGG 3' and SKC-II as primer 2 with the sequence 5' GAAGGCCTTCCCCT TTGAAA 3' according to Rosey *et al*. [51]. The PCR temperature program consisted of 30 cycles: 94°C for 60 sec, 52°C for 60 sec and 72°C for 90 sec. The amplification of *pauA* gene was performed with the oligonucleotide primer 1 P38 5' AATAACCGGT TATGATTCCGACTAC 3' and primer 2 P39 5' AAAATTTACTCGAGACTTCCTT TAAGG 3' described by Johnsen *et al*. [34]. The thermal cycler program consisted of 30 cycles: 94°C for 60 sec, 54°C for 60 sec and 72°C for 90 sec. The final cycle was followed by an extension incubation at 72°C for 5 min, respectively. The PCR products was determined by electrophoresis of 12 µl of the reaction product in a 2% (w/v) agarosegel (Sigma) with Tris acetate-electrophoresis buffer (TAE) (4.0 mmol/l Tris, 1 mmol/l EDTA, (pH 7.8) and a 100 bp DNA ladder (Gibco BRL, Eggenstein, Germany) as molecular marker.

Finally a macrorestriction analysis of the chromosomal DNA of the cultures was performed according to Soedarmanto *et al*. [62]. The DNA-fingerprinting was carried out by preparation of whole bacterial DNA of the isolates in agarose gel plugs and subsequent digestion of the bacterial DNA with the restriction enzyme *Sma*I and separation of the fragments by PFGE using the pulse time described by Baseggio *et al*. [7]. The interpretation of the restriction patterns was performed as described by Tenover *et al*. [64].

Results

All 131 streptococci investigated in the present study were Gram positive chain forming cocci. The somatic cell count analysis of the corresponding milk samples revealed the subclinical status of mastitis for 126 of the 131 selected samples. However, five of the samples exhibited a clinical status of mastitis. By cultivation on sheep blood agar 126 isolates were α-hemolytic while the remaining five strains were non-hemolytic. After cultivation on Columbia esculin blood agar, all 131 isolates degraded esculin. None of the 131 isolates grew on CATC and KAA media. However, nine, seven and four isolates showed a weak growth on Esculin bile agar, Chromocult enterococci agar and Slanetz-Bartley agar, respectively. The reference strains of both species could not be cultivated on all five media specific for enterococci.

All 131 isolates and the four reference strains exhibited degradation of fructose, glucose, maltose, mannitol, saccharose, salicin, sorbitol and trehalose and hydrolyzed esculin and sodium hippurate, while all isolates did not ferment arabinose. All 135 isolates except one fermented lactose, ribose and hydrolysed arginine, respectively. Among the 135 streptococci investigated 94 and 4 of the strains fermented inulin and raffinose, respectively.

Additionally, 130 of the investigated strains, and the *S. uberis* reference strains NCDO 2038 and NCDO 2086 showed β-D-glucuronidase enzyme activity whereas isolate 138/80 and the *S. parauberis* strains NCDO 2020 and 94/16 were negative in this enzyme. Investigating pyrrolidonyl aminopeptidase enzyme activities 120 isolates yielded a positive reaction. Hyaluronidase enzyme activity, demonstrated by forming non-mucoid colonies of the mucoid growing *S. equi* subsp. *zooepidemicus* indicator strain, could be observed for 47 of the 135 isolates. The remaining strains, also including the reference strains of both species, appeared to be hyaluronidase negative.

Among the 131 strains investigated, 130 strains were classified as *S. uberis* and strain 138/80 as *S. parauberis*.

The serological investigations revealed that 42 of the 130 *S. uberis* strains, *S. uberis* reference strain NCDO 2038 and *S. parauberis* reference strain NCDO 2020, reacted with group E specific antiserum, whereas 11 and 9 of the 130 *S. uberis* strains reacted with group P and group U specific antiserum, respectively. One of the *S. uberis* isolates reacted with group A, *S. uberis* reference strain NCDO 2086 with group G, one *S. uberis* strain

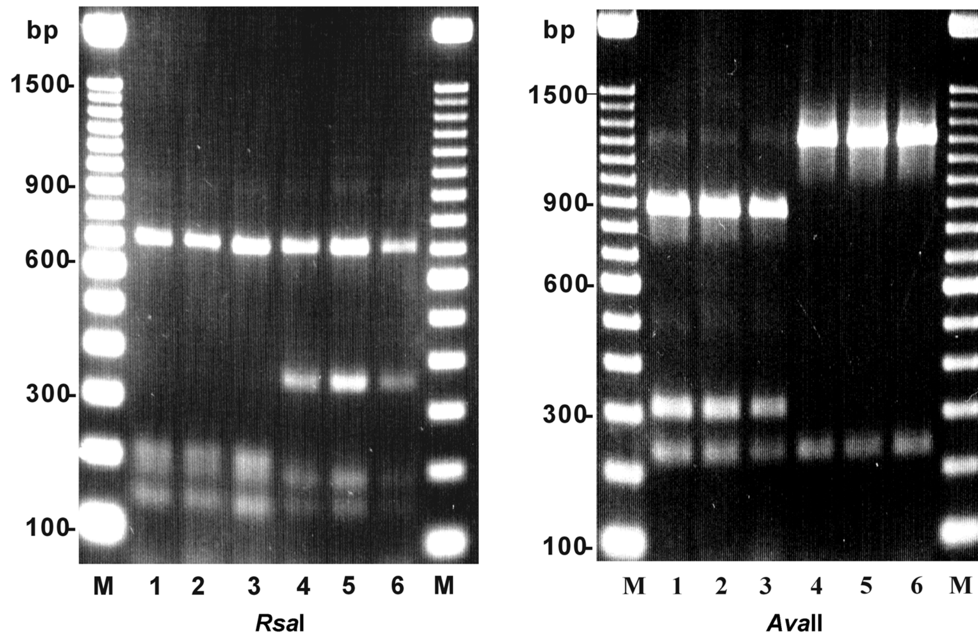


Fig. 1. Typical fragments of the PCR amplified 16S rRNA gene of *S. uberis* (1, 2, and 3) and *S. parauberis* (4, 5, and 6) after digestion with the restriction enzymes *Rsa*I and *Ava*II, respectively. M = a 100 bp ladder size marker.

simultaneously with group E and group P and one *S. uberis* with group E and group U specific antisera, respectively. The remaining 65 *S. uberis* strains, *S. parauberis* 138/80 and *S. parauberis* 94/16 were categorized as non-groupable.

A synergistic hemolytic CAMP-like reaction on sheep blood agar within the zone of staphylococcal α-toxin could be observed for five of the 130 *S. uberis* strains. In lectin agglutination reactions, 43 of 130 *S. uberis* strains exhibited agglutination reactions with the lectin of Helix pomatia. A self-agglutination reaction was observed for two *S. uberis* strains even after trypsin pretreatment of the bacteria. None of the remaining *S. uberis* and *S. parauberis* strains also including the reference strains of both species showed a comparable reaction with the lectin investigated.

A molecular characterization of the bacteria was performed by RFLP of the 16S rRNA gene. All 131 strains investigated and the four reference strains of both species displayed an amplicon size of the 16S rRNA gene of 1430 bp. The amplified 16S rRNA gene was digested with the restriction endonucleases *Rsa*I and *Ava*II, respectively. The restriction profiles confirmed the classification of 130 strains as *S. uberis* and strain 138/80 as *S. parauberis*. Identical restriction profiles could be observed for the reference strains of both species, respectively. *Rsa*I restriction of the *S. uberis* 16S rRNA gene revealed four fragments with sizes of approximately 140, 190, 220 and 700 bp and for the *S. parauberis* 16S rRNA gene four fragments with sizes of approximately 140, 190, 380 and 700 bp. *Ava*II restriction revealed three different fragments with sizes of 230, 310 and 900 bp for *S. uberis* and fragment sizes of 230 and 1,200 bp for *S. parauberis* (Fig. 1).

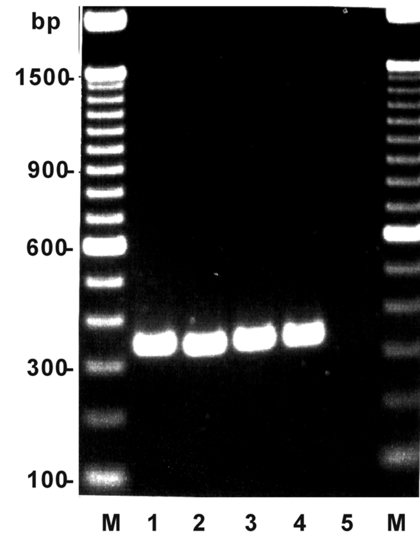


Fig. 2. Amplicons of *S. uberis* (1, 2, 3, 4) with a size of 340 bp using the *S. uberis* 16S-23S rDNA intergenic spacer region specific oligonucleotide primers; *S. parauberis* (5) served as negative control. M = see Fig. 1.

Using oligonucleotide primers amplifying *S. uberis* specific parts of the 16S rRNA gene, the 23S rRNA gene and the 16S-23S rDNA intergenic spacer region revealed amplicons with sizes of 440, 450 and 340 bp, respectively. This could be observed for all 130 *S. uberis* and both *S. uberis* reference strains but not for *S. parauberis*. Typical

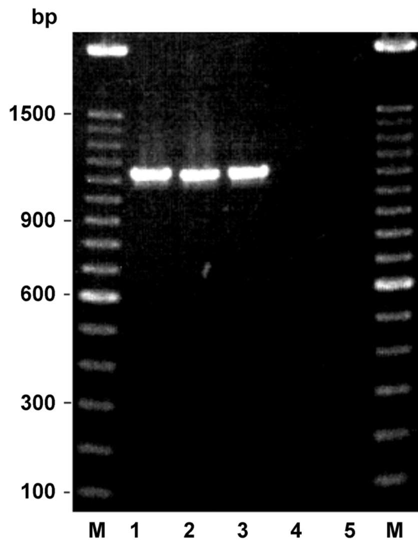


Fig. 3. Amplicons of *S. uberis* (1, 2, 3) with a size of 1130 bp using the *S. uberis skc* gene specific primers SKC-I and SKC-II; *skc* gene negative *S. uberis* and *S. parauberis* are shown in lane 4 and 5. M = see Fig. 1.

amplicons using *S. uberis* 16S-23S rDNA intergenic spacer region specific oligonucleotide primers are shown in Fig. 2. For *S. parauberis* species specific parts of the 16S rRNA gene, the 23S rRNA gene and the 16S-23S rDNA intergenic spacer region with sizes of 880, 480 and 200 bp, respectively, could be observed (data not shown).

Using oligonucleotide primers specific for CAMP factor gene *cfu* an amplicon with a size of 680 bp could be observed for five *S. uberis* strains. All five strains positive for gene *cfu* were also phenotypically CAMP positive. Selected phenotypically CAMP negative *S. uberis* (n = 31) and all three phenotypically CAMP negative *S. parauberis* strains were also genotypically negative. Investigating the 130 *S. uberis*, *S. parauberis* 138/80 and the four reference strains of both species for gene *skc* a specific amplicon with a size of 1130 bp could be observed for 126 of the investigated *S. uberis* and both *S. uberis* reference strains (Fig. 3). The remaining four *S. uberis* and the three *S. parauberis* strains were negative. Investigating the strains for gene *pauA* a specific amplicon with a size of 800 bp could be observed for all 128 *skc* positive *S. uberis* strains. No amplicon could be observed for the remaining strains. Some phenotypical and genotypical characteristics are summarized in Table 1.

For DNA fingerprinting, a macrorestriction analysis of the chromosomal DNA of the bacteria was determined by PFGE. This was performed with 69 arbitrarily selected strains obtained from 57 cows of 26 different farms. Digestion of the chromosomal DNA of the isolates was performed with the endonuclease *Sma*I. The 69 selected strains displayed 55 different DNA patterns. Identical PFGE patterns could be observed for some of the isolates within different quarters of an individual cow and between different cows within the same farm. The PFGE patterns of 9 isolates from five different cows of farm 2 are shown in Fig. 4.

Discussion

S. uberis is important to the veterinary domain because of its increasing association with bovine mastitis. The *S. uberis* mastitis causes a tremendous economic loss in milk production and has become the major environmental mastitis agent [66].

The present results strongly support the findings described by Lerondelle [42] that a *S. uberis* infection rarely gives rise to clinical mastitis. The infection remains subclinical during long periods of time. In the absence of treatment, this causes serious losses in milk production. Also corresponding to the present work, Bramley [9] and Jayarao *et al.* [32] described a high prevalence of subclinical forms of *S. uberis* intramammary infections in dairy cows. According to these authors, a *S. uberis* subclinical mastitis frequently occurs before parturition and near drying-off period, whereas a clinical mastitis with *S. uberis* could be observed more frequently in the first five weeks of lactation.

The esculin positive bacteria of the present investigation were further cultivated on five different media selective for enterococci. The growth patterns of the cultures were clearly different to a comparatively cultivated *E. faecalis* strain indicating that all five media could be used to differentiate between esculin degrading enterococci and *S. uberis*.

According to hemolysis on blood agar plate and the carbohydrate fermentation tests, all strains displayed, comparable to various authors [16,17,19,23,56,68], the typical properties of *S. uberis* and *S. parauberis.*

However, according to Williams and Collins [69] and Doménech *et al.* [19] and the results of the present study the enzyme β-D-glucuronidase seems to be the only criterion allowing a differentiation of *S. uberis* and *S. parauberis*.

Serogrouping of the bacteria revealed that 42 isolates and one *S. uberis* and *S. parauberis* reference strain were positive with group E specific antisera, some strains were positive with group A, G, P and U specific antisera alone or in combination. Comparable to the present studies Lämmler [36] and Roguinsky [49,50] also reported that *S. uberis* strains could serologically be classified into Lancefield group E, P, G and U. Some of the strains investigated by Roguinsky [49,50] simultaneously reacted with group E and group P, group P and group U, group P and group G specific antiserum, respectively; some strains were serologically non-groupable. A reaction of some *S.*

Table 1. Some pheno- and genotypic characteristics of 132 *S. uberis* and 3 *S. parauberis*

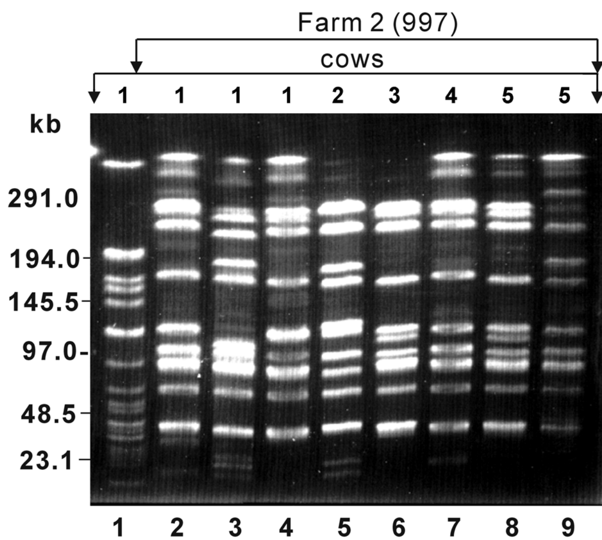
		*S. uberis** (n=132)	*S. parauberis*** (n=3)
Growth on	CATC KAA	no growth	no growth
	Esculin bile agar	9[1]	
	Chromocult enterococci agar	7[1]	
	Slanetz-Bartly agar	4[1]	
Haemolysis	alpha	126[2]	-
	non	6	3
Carbohydrate fermentation	arabinose	-	-
	fructose, glucose, maltose, mannitol, saccharose, salicin, sorbitol, trehalose	132	3
	lactose, ribose	131	3
	Inulin	92	2
	raffinose	2	2
Hydrolysis of	esculin	132	3
	hippurate	132	3
	arginine	131	3
Enzyme activities	β-D-glucuronidase	132	-
	Pyrrolidonyl aminopeptidase	117	3
	Hyaluronidase	47	-
Serogrouping	E	43	1
	P	11	-
	U	9	-
	A	1	-
	G	1	-
	E and P	1	-
	E and U	1	-
	non-groupable	65	2
Lectin agglutination	Helix pomatia	43	-
CAMP-like factor	sheep blood agar plates	5	-
Specific PCR reaction	*cfu* gene	5	-
	skc gene	128	-
	pauA gene	128	-

*including *S. uberis* reference strains NCDO 2038 and NCDO 2086
**including *S. parauberis* reference strain NCDO 2020, strain 94/16 and strain 138/80
[1]weak growth
[2]number of strains showing a positive reaction
-negative reaction

uberis with group P, G, U and B specific antisera had also been reported by other authors [12,23,26,54,55]. Among the three *S. parauberis* strains reference strain NCDO 2020 reacted with group E specific antiserum whereas the remaining two strains were non-groupable.

Lectin agglutination reactions were conducted with the lectin from Helix pomatia. Corresponding to Niewerth *et al.* [43], Lämmler [36], Christ and Lämmler [13] and Abdulmawjood *et al.* [1] some *S. uberis* of the present study specifically reacted with the lectin from Helix pomatia indicating the usefulness of lectin agglutination reactions to phenotypically characterize bacteria of this species.

A molecular identification of both species could be performed by RFLP analysis of the 16S rRNA gene. Corresponding to Jayarao *et al.* [31], as well as Lämmler *et al.* [38] and Hassan *et al.* [27] all *S. uberis* and *S. parauberis* strains of the present investigation showed a specific restriction profile using the restriction enzymes *Rsa*I and *Ava*II. RFLP analysis of the 16S rRNA gene had



* number of cows

Fig. 4. Pulsed-field gel electrophoretic restriction patterns of chromosomal DNA of 9 *S. uberis* isolated from 5 cows of a single farm using the restriction enzyme *Sma*I. Six different DNA restriction patterns were observed; pattern I (lane 1), pattern II (lane 2, 4, 7), pattern III (lane 3), pattern IV (lane 5), pattern V (lane 6, 8) and pattern VI (lane 9).

already been used for characterization of *S. agalactiae* and *S. porcinus* [2,39]. Comparable to the present results, these authors also found no intraspecies variations for the 16S rRNA genes of *S. agalactiae* and the serologically heterogenous species *S. porcinus*. However, an intraspecies variation in the sequence of the 16S rRNA gene was observed for *S. suis* [11] and for *S. equi* subsp. *zooepidemicus* [4].

In further studies, a PCR-based identification with specific oligonucleotide primers targeted to species-specific regions of the gene encoding the 16S rRNA, the gene encoding the 23S rRNA and the 16-23S rDNA intergenic spacer region of *S. uberis* and *S. parauberis* respectively, was performed. All three target genes could successfully be used to identify and differentiate both species. Comparable investigations were carried out by Forsman *et al.* [22] investigating *S. uberis* specific parts of the 16-23S rDNA intergenic spacer region and by Hassan *et al.* [28] using *S. uberis* and *S. parauberis* specific regions of the genes encoding the 16S rRNA and the 23S rRNA, and *S. parauberis* specific regions of the 16S-23S rDNA intergenic spacer region. In addition, Tilsala-Timisjärvi *et al.* [65] used species-specific oligonucleotide primers targeted to the 16-23S rDNA intergenic spacer region for differentiation of *S. uberis* and other pathogenic streptococcal and staphylococcal species. Moreover, Phuektes *et al.* [45] used a 16-23S rDNA intergenic spacer region based multiplex PCR assay for identification and differentiation of *S. uberis* and other mastitis pathogens. Similarly, Riffon et al. [48] described species-specific parts of the 23S rRNA gene and the 16-23S rDNA intergenic spacer region of *S. uberis* as well as species-specific parts of the 23S rRNA gene of *S. parauberis*.

An additional potential virulence factor investigated in the present study was the CAMP factor and the CAMP factor encoding gene *cfu*. The importance of "uberis factor" for the virulence of *S. uberis* has been pointed out by Skalka and Smola [60]. These authors parenterally administered an "uberis factor" containing exosubstance of *S. uberis* to rabbits and white mice causing the death of the animals. In 1979, Skalka *et al.* [59] reported that 58 of 81 investigated *S. uberis* strains produced a hemolytically active exosubstance showing an identical effect as the CAMP factor of *S. agalactiae*. Similarly, Christ *et al.* [12] and Lämmler [36] found 10% and 28% CAMP positive *S. uberis* strains, respectively. A positive CAMP-reaction and the detection of gene *cfu* could be observed for five *S. uberis* of the present investigation. The latter could be demonstrated, with oligonucleotide primers designed in the present study. In 1996 Jiang *et al.* [33] cloned and sequenced the CAMP factor gene *cfu*. According to these authors the CAMP factor gene cfu of *S. uberis* and the deduced amino acid sequence appeared to be highly homologous to the *cfb* gene and amino acid sequence of *S. agalactiae*. Similarly, Gase *et al.* [24] described a sequence homology of CAMP factor gene *cfa* of group A streptococci, *cfb* of group B streptococci and *cfu* of *S. uberis*. These authors also suggested that CAMP factor and CAMP factor-like genes are fairly widespread among streptococci, at least in serogroups A, B, C, G, M, P, R and U. In addition, Hassan *et al.* [27] found a close relation of the CAMP gene *cfa* of *S. pyogenes*, *cfb* of *S. agalactiae*, *cfu* of *S. uberis* and *cfg* of *S. canis*.

An additional potential virulence gene investigated in the present study was the gene pauA/skc encoding a plasminogen activator. According to previous investigations, bovine plasminogen activated by streptokinase seemed to be a virulence factor of *S. uberis* during early stages of infection. This activation might cause a rapid growth of the bacteria in the lactating bovine mammary gland [40]. For *S. uberis* the plasminogen activator gene pauA and the plasminogen activator gene designated as streptokinase gene skc was cloned and sequenced by Rosey *et al.* [51] and Johnsen *et al.* [34], respectively. According to Leigh [41] the gene pauA was produced by the majority of the *S. uberis* strains isolated from clinical cases of bovine mastitis. Comparable to these findings most of the *S. uberis* of the present investigation were pauA and skc positive. However, comparing the sequence of both genes revealed their complete sequence identity (data not shown).

To determine the possibly existing epidemiological relationship of the collected *S. uberis* strains of the present

investigation, a macrorestriction analysis of the chromosomal DNA of arbitrarily selected *S. uberis* was performed by PFGE. DNA macrorestriction analysis by PFGE is an essential tool for epidemiological investigations to identify specific strains of a causative bacterial species as well as for the comparison of strains between cows and farms, and has already successfully been used to investigate restriction patterns among strains of *S. uberis* [7]. Gordillo *et al.* [25] used PFGE for typing group B streptococci and described that PFGE patterns could easily be discerned, interpreted and potentially utilized for epidemiological investigations.

The isolates of the present study were collected from bovine milk of a defined area within a time period of three months. This collection corresponded to the criteria of epidemiological isolates proposed by Tenover *et al.* [64]. These authors additionally defined a set of guidelines for interpreting DNA restriction patterns generated by PFGE and for using these results as epidemiologically useful information.

The PFGE restriction patterns obtained from 69 selected *S. uberis* strains were comparatively investigated after digestion with the endonuclease *Sma*I. The results of the present study revealed mostly nonidentical PFGE patterns. However, for some strains identical PFGE patterns could be observed for isolates within different quarters of an individual cow and different cows within the same farm. Among 69 *S. uberis* strains isolated from 57 cows from 26 different farms 55 different DNA restriction patterns were observed, indicating that a wide variety of *S. uberis* strains might infect and cause mammary gland infection due to the contamination of the gland from the environment. This high degree of heterogeneity supports the epidemiological studies by Baseggio *et al.* [7], also suggesting a limited transmission of infection from cow-to-cow during milking process. These authors examined and differentiated *S. uberis*, *S. agalactiae* and *S. dysgalactiae* isolates by PFGE also after digestion with the restriction enzyme *Sma*I. The *S. uberis* isolates investigated in these studies displayed diverse restriction patterns. However, the investigated *S. dysgalactiae* had most diverse and complex restriction patterns. In contrast to the latter the species *S. agalactiae* had identical restriction patterns within the herds but distinct between herds. The studies of Douglas *et al.* [20] additionally supported the results of the macrorestriction analysis of the *S. uberis* isolates of the present investigation. According to these authors 330 different PFGE patterns could be observed from 343 isolates. In addition Wang *et al.* [67] reported that S. uberis had most diverse PFGE patterns as compared to *S. agalactiae* and *S. dysgalactiae*. According to these authors, 74 distinct PFGE patterns could be observed among 130 *S. uberis* strains collected from 73 different cows of 3 farms. In contrast to *S. uberis*, the *S. agalactiae* isolates examined by Wang *et al.* [67] exhibited, corresponding to the results of Baseggio *et al.* [7], identical patterns within the same farm but different patterns between various farms. The latter indicated that a single clone was transmitted between cows. Fink *et al.* [21] also analysed and compared macrorestriction patterns of *S. agalactiae* isolated from bovine mastitis. According to these authors, also a single clone seemed to be responsible for the mastitis situation within a herd. The clones differed between herds. Moreover, Annemüller *et al.* [6] analysed PFGE patterns of *Staphylococcus aureus* strains isolated from cows with mastitis. These studies revealed that isolates from a single farm generally had identical restriction patterns. This could also be observed for isolates of different herds. Akineden *et al.* [5] also described that *S. aureus* had identical PFGE restriction patterns within the same farm but different patterns between the farms investigated.

Despite the degree of heterogeneity in DNA restriction patterns, some *S. uberis* strains of the present study isolated from a single cow as well as from different cows of the same farm displayed identical PFGE patterns, indicating that some *S. uberis* strains might be transmitted from quarter to quarter and cow to cow of a single farm. This also corresponded to the findings of Phuektes *et al.* [46]. These authors investigated the epidemiological status of *S. uberis* mastitis in dairy cows and detected nonidentical and also identical PFGE patterns.

According to the results of the present study, a macrorestriction analysis of the *S. uberis* isolates by PFGE appears to be a useful and reliable method to study the epidemiological relationship of the investigated strains.

The conventional and molecular methods used in the present study allowed a reliable identification and further characterization of *S. uberis* and *S. parauberis* and might help investigate the importance of both species as causative agents of bovine mastitis. However, according to the present results, the occurrence of *S. parauberis* as mastitis causing pathogen seems to be rare.

References

1. **Abdulmawjood, A., Soedarmanto, I. and Lämmler, C.** Identification of streptococci of serological group C using fluorescein and peroxidase conjugated lectins. Med. Sci. Res. 1996, **24**, 375-376.
2. **Abdulmawjood, A., Weiß, R. and Lämmler, C.** Species identification of *Streptococcus porcinus* by restriction fragment length polymorphism analysis of 16S ribosomal DNA. Res. Vet. Sci. 1998, **65**, 85-86.
3. **Abdulmawjood, A. and Lämmler, C.** Amplification of 16S ribosomal RNA gene sequences for the identification of streptococci of Lancefield group B. Res. Vet. Sci. 1999, **67**, 159-162.
4. **Abdulmawjood, A. and Lämmler, C.** 2000. Determination of intraspecies variations of the V2 region of the 16S rDNA

gene of *Streptococcus equi* subsp. *zooepidemicus*. Res. Vet. Sci. 2000, **68**, 33-39.

5. **Akineden, Ö., Annemüller, C., Hassan, A. A., Lämmler, C., Wolter, W. and Zschöck, M.** Toxin genes and other characteristics of *Staphylococcus aureus* isolates from milk of cows with mastitis. Clin. Diagn. Lab. Immunol. 2001, **8**, 959-964.
6. **Annemüller, C., Lämmler, C. and Zschöck, M.** Genotyping of *Staphylococcus aureus* isolated from bovine mastitis. Vet. Microbiol. 1999, **69**, 217-224.
7. **Baseggio, N., Mansell, P. D., Browning, J. W. and Browning, G. F.** Strain differentiation of isolates of streptococci from bovine mastitis by pulsed-field gel electrophoresis. Mol. Cell. Probes. 1997, **11**, 349-354.
8. **Bentley, R. W. and Leigh, J. A.** Development of the PCR-based hybridization protocol for identification of streptococcal species. J. Clin. Microbiol. 1995, **33**, 1296-1301.
9. **Bramley, A. J.** *Streptococcus uberis* udder infection-a major barrier to reducing mastitis incidence. Beecham Mastitis Series, Shinfield, Reading RG.1980, **29AT**, 328-335.
10. **Bramley, A. J.** Source of *Streptococcus uberis* in the dairy herd. Isolation from bovine feces and from straw bedding of cattle. J. Dairy Res. 1982, **49**, 369-373.
11. **Chatellier, S., Harel, J., Zhang, Y., Gottschalk, M., Higgins, R., Devriese, L. A. and Brousseau, R.** Phylogenetic diversity of *Streptococcus suis* strains of various serotypes as revealed by 16S rDNA gene sequence comparison. Int. J. Syst. Bacteriol. 1998, **48**, 581-589.
12. **Christ D., Schwarz, S. and Lämmler, C.** DNA-fingerprinting of *Streptococcus uberis*. Med. Sci. Res. 1988, **16**, 1297-1298.
13. **Christ, D. and Lämmler, C.** Characterization of *Streptococcus uberis* with special consideration of supposed pathogenicity factors. Berl. Münch. Tierärztl. Wschr. 1992, **105**, 224-230.
14. **Collins, M. D., Farrow, J. A. E., Katic, V. and Kandler, O.** Taxonomic studies on streptococci of serological group E, P, U and V: Description of *Streptococcus porcinus* sp. nov. Syst. Appl. Microbiol. 1984, **5**, 402-413.
15. **Cullen, G. A.** The ecology of *Streptococcus uberis*. Br. Vet. J. 1966, **122**, 333-339.
16. **Cullen, G. A.** *Streptococcus uberis* and its relationship to bovine mastitis. Univ. of London. Ph.D. Thesis. 1968.
17. **Cullen, G. A.** Isolation of *S. uberis* from lactating and non-lactating cows. Br. Vet. J. 1969, **125**, 145-148.
18. **Daleel, E. E. and Frost, A. J.** Some observations on the bacterial flora of the bovine tonsil. Br. Vet. J. 1967, **123**, 232-236.
19. **Doménech, A., Fernández-Garayzábal, J. F., Pascual, C., Garcia, J. A., Cutuli, M.T., Moreno, M. A., Collins, M. D. and Dominguez, L.** Streptococcosis in cultured turbot, *Scophthalmus maximus* (L.), associated with *Streptococcus parauberis*. J. Fish Dis. 1996, **19**, 33-38.
20. **Douglas, V. L., Fenwick, S. G., Pfeiffer, D. U., Williamson, N. B. and Holmes, C. W.** Genomic typing of *Streptococcus uberis* isolates from cases of mastitis, in New Zealand dairy cows, using pulsed-field gel electrophoresis. Vet. Microbiol. 2000, **75**, 27-41.
21. **Fink, K., Abdulmawjood, A., Lämmler, C. and Zschöck, M.** Genotypisierung von *Streptococcus agalactiae*-Kulturen, isoliert von subklinischen Rindermastitiden. 41 Arbeitstagung des Arbeitsgebietes “Lebensmittelhygiene” der Deutschen Veterinärmedizinischen Gesellschaft in Garmisch-Partenkirchen. Tagungsbericht. pp. 177-182, 2000.
22. **Forsman, P., Tilsala-Timisjärvi, A. and Alatossava, T.** Identification of staphylococcal and streptococcal causes of bovine mastitis using 16S-23S rDNA spacer regions. Microbiol. 1997, **143**, 3491-3500.
23. **Garvie, E. I. and Bramley, A. J.** *Streptococcus uberis*: an approach to its classification. J. Appl. Bacteriol. 1979, **46**, 295-304.
24. **Gase, K., Ferretti, J. J., Primeaux, C., McShan, M. W.,** Identification, cloning, and expression of the CAMP factor gene (*cfa*) of group A streptococci. Infect. Immun. 1999, **67**, 4725-4731.
25. **Gordillo, M. E., Singh, K. V., Baker, C. J. and Murray, B. E.** Typing of group B streptococci: comparison of pulsed-field gel electrophoresis and conventional electrophoresis. J. Clin. Microbiol. 1993, **31**, 1430-1434.
26. **Hahn, G.** Ergebnisse aus der Streptokokken-Zentrale in Kiel von 1965-1978: Mastitis-Streptokokken. Zbl. Bakt. Hyg. I. Abt. Orig. 1981, **A249**, 223-340.
27. **Hassan, A. A., Abdulmawjood, A., Yildirim, A. Ö., Fink, K., Lämmler, C. and Schlenstedt, R.** Identification of streptococci isolated from various sources by determination of *cfb* gene and other CAMP-factor genes. Can. J. Microbiol. 2000, **46**, 946-951.
28. **Hassan, A. A., Khan, I. U., Abdulmawjood, A. and Lämmler, C.** Evaluation of PCR methods for the rapid identification and differentiation of *Streptococcus uberis* and *Streptococcus parauberis*. J. Clin. Microbiol. 2001, **39**, 1618-1621.
29. **Hwang, M. N. and Ederer, G. M.** Rapid hippurate hydrolysis for presumptive identification of group B-streptococci. J. Clin. Microbiol. 1975, **30**, 2235-2240.
30. **Jayarao, B. M., Doré, Jr. J. J. E., Baumbach, G. A., Matthews, K. R. and Oliver, S. P.** Differentiation of *Streptococcus uberis* from *Streptococcus parauberis* by polymerase chain reaction and restriction fragment length polymorphism analysis of the 16S ribosomal DNA. J. Clin. Microbiol. 1991, **29**, 2774-2778.
31. **Jayarao, B. M., Doré, Jr. J. J. E., and Oliver, S. P.** Restriction fragment length polymorphism analysis of 16S ribosomal DNA of *Streptococcus* and *Enterococcus* species of bovine origin. J. Clin. Microbiol. 1992, **30**, 2235-2240.
32. **Jayarao, B. M., Gillespie, B. E., Lewis, M. J., Dowlen, H. H. and Oliver, S. P.** Epidemiology of *Streptococcus uberis* intramammary infections in a dairy herd. J. Vet. Med. 1999, **46**, 433-442.
33. **Jiang, M., Babiuk, L. A. and Potter. A. A.** Cloning, sequencing and expression of the CAMP factor gene of *Streptococcus uberis*. Microb. Pathol. 1996, **20**, 297-307.
34. **Johnsen, L. B., Poulsen, K., Kilian, M. and Peterson, T. E.** Purification and cloning of a streptokinase from *Streptococcus uberis*. Infect. Immun. 1999, **67**, 1072-1078.

35. **King, J. S.** *Streptococcus uberis*: a review of its role as a causative organism of bovine mastitis. II. Control of infection. Br. Vet. J. 1981, **137**, 160-165.
36. **Lämmler, C.** Biochemical and serological properties of *Streptococcus uberis*. J. Vet. Med. 1991, **38**, 737-742.
37. **Lämmler, C. and Hahn, G. Streptokokken In: Blobel, H. und Schließer, T.** (eds): Handbuch der bakteriellen Infektionen bei Tieren. Band II/2: Streptokokken-Infektionen und Rotlauf. 2. Auflage. Gustav Fischer Verlag, Jena/ Stuttgart. 1994.
38. **Lämmler, C., Abdulmawjood, A., Danic, G., Vaillant, S. and Weiß, R.** Differentiation of *Streptococcus uberis* and *Streptococcus parauberis* by restriction fragment length polymorphism analysis of the 16S ribosomal RNA gene and further studies on serological properties. Med. Sci. Res. 1998, **26**, 177-179.
39. **Lämmler, C., Abdulmawjood, A. and Weiß, R.** Properties of serological group B streptococci of dog, cat and monkey origin. J. Vet. Med. 1998, **45**, 561-566.
40. **Leigh, J. A.** Activation of bovine plasminogen by *Streptococcus uberis*. FEMS Microbiol. Lett. 1993, **114**, 67-72.
41. **Leigh, J. A.** Purification of a plasminogen activator from *Streptococcus uberis*. FEMS Microbiol. Lett. 1994, **118**, 153-158.
42. **Lerondelle, C.** Les mammites a *Streptococcus uberis*. Res. Méd. Vét. Ec. Alfort. 1985, **161**, 539-744.
43. **Niewerth, B.** Wechselwirkung zwischen Gram-positiven Bakterien, Lektinen und Körperzellen. Vet. Med. Diss., Justus-Liebig-Universität Giessen. Ph.D. Thesis. 1987.
44. **Obiger, G.** Untersuchungen über die in Rindertonsillen vorkommenden Streptokokken. Kieler Milchwirtschaftl. Forschungsber. 1954, **6**, 595-603.
45. **Phuektes, P., Mansell, P. D. and Browning, G. F.** Multiplex polymerase chain reaction assay for simultaneous detection of *Staphylococcus aureus* and streptococcal causes of bovine mastitis. J. Dairy Sci. 2001, **84**, 1140-1148.
46. **Phuektes, P., Mansell, P. D., Dyson, R. S., Hooper, N. D., Dick, J. S. and Browning, G. F.** Molecular epidemiology of *Streptococcus uberis* isolates from dairy cows with mastitis. J. Clin. Microbiol. 2001, **39**, 1460-1406.
47. **Rantz, L. A. and Randall, E.** Use of autoclaved extracts of hemolytic streptococci for serological grouping. Stanford Med. Bull. 1955, **13**, 290-291.
48. **Riffon, R., Sayasith, K., Khalil, H., Dubreuil, P., Drolet, M. and Lagace, J.** Development of a rapid and sensitive test for identification of major pathogens in bovine mastitis by PCR. J. Clin. Microbiol. 2001, **39**, 2584-2589.
49. **Roguinsky, M.** Reactions de *Streptococcus uberis* avec les serums G et P. Ann. Inst. Pasture. 1969, **117**, 529-532.
50. **Roguinsky, M.** Caracteres biochimiques et serologiques de *Streptococcus uberis*. Ann. Inst. Pasture. 1971, **126**, 529-532.
51. **Rosey, E. L., Lincoln, R. A., Ward, P. N., Yancey, Jr. R. J. and Leigh, J. A.** PauA: a novel plasminogen activator from *Streptococcus uberis*. FEMS Microbiol. Lett. 1999, **178**, 27-33.
52. **Schalm, O. W., Carroll, E. J. and Jain, N. C.** In bovine mastitis. Lee and Febiger. Philadelphia. PA. USA. 1971.
53. **Schleifer, K. H. and Kilpper-Bälz, R.** Molecular and chemotaxonomic approaches to the classification of streptococci, enterococci and lactococci: A review. Syst. Appl. Microbiol. 1987, **10**, 1-19.
54. **Schuman, R. D., Nord, N., Brown, R. W., Wessman, G. E. and Wessman, G. E.** Biochemical and serological characteristics of Lancefield groups E, P, and U streptococci and *Streptococcus uberis*. Cornell Vet. 1972, **62**, 540-562.
55. **Schuman, R. D., Wood, R. L. and Nord, N.** Swine abscesses caused by Lancefields group E streptococci. X. Specificity of the precipitin test for their detection with relation to the streptococcal antigens of groups R and T, *Streptococcus uberis* and *Staphylococcus aureus*. Cornell Vet. 1972, **62**, 61-73.
56. **Seeley, H. W.** The physiology and nutrition of *Streptococcus uberis*. J. Bacteriol. 1951, **62**, 107-115.
57. **Sharma, R. M. and Parker, R. A.** Occurrence and ecologic features of *Streptococcus uberis* in the dairy cows. Am. J. Vet. Res. 1970, **31**, 1197-1202.
58. **Sherman, J. M.** The streptococci. Bacteriol. Rev. 1937, **1**, 3-97.
59. **Skalka, B., Smola, J. and Pilich, J.** Diagnostic availability of the hemolytically active exosubstance of *Corynebacterium pseudotuberculosis* for isolation and identification of *Streptococcus agalactiae* and its comparison with the beta-toxin of *Staphylococcus aureus*. Zbl. Vet. Med. 1979, **B26**, 679-687.
60. **Skalka, B. and Smola, J.** Lethal effect of CAMP-factor and Uberis-factor- a new finding about diffusible exosubstances of *Streptococcus agalactiae* and *Streptococcus uberis*. Zbl. Bakt. Hyg. I. Abt. Orig. 1981, **A249**, 190-194.
61. **Slot, P. A.** Classification, systemics and occurrence. Nord. Vet. Med. 1958, **10**, 143-152.
62. **Soedarmanto, I., Pasaribu, F. H., Wibawan, I. W. and Lämmler C.** Identification and molecular characterization of serological group C streptococci isolated from diseased pigs and monkeys in Indonesia. J. Clin. Microbiol. 1996, **34**, 2201-2204.
63. **Sweeney, E. J.** Observation on the epidemiology of mastitis due to *Streptococcus uberis* in a laboratory herd during a complete lactation. Res. Vet. J. 1964, **5**, 483-487.
64. **Tenover, F. C., Arbeit, R. D., Goering, R. V., Mickelsen, P. A., Murray, B. E., Persing, D. H. and Swaminathan, B.** Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J. Clin. Microbiol. 1995, **33**, 2233-2239.
65. **Tilsala-Timisjärvi, A., Forsman, P. and Alatossava, T.** Bovine mastitis diagnosis from milk by a polymerase chain reaction-based reaction. J. Nutri. Res. Food Sci. 2000, **55**, 488-492.
66. **Todhunter, D. A., Smith, K. L. and Hogan, J. S.** Environmental streptococcal mastitis. In: Proc. pp. 92-95, Natl. Mast. Counc., Arlington. 1994.
67. **Wang, S. M., Deighton, M. A., Capstick, J. A. and Gerraty, N.** Epidemiological typing of bovine streptococci by pulsed-field gel electrophoresis. Epidemiol. Infect. 1999, **123**, 317-324.
68. **Williams, A. M. and Collins, M. D.** Molecular taxonomic

studies on *Streptococcus uberis* types I and II. Description of *Streptococcus parauberis* sp. nov. J. Appl. Bacteriol. 1990, **68**, 485-490.

69. **Williams, A. M. and Collins, M. D.** DNA fingerprinting of *Streptococcus uberis* based on polymorphism of DNA encoding rRNA. Lett. Appl. Microbiol. 1991, **12**, 23-28.

70. **Winkle, S.** Mikrobiologische und Serologische Diagnostik. 3. Auflage, Gustav Fischer Verlag, Stuttgart/New York. 1979.